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Research paper

Stabilization of all-*trans* retinol by loading lipophilic antioxidants in solid lipid nanoparticles

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Abstract

Loading of drugs into the solid matrix of solid lipid nanoparticles (SLNs) can be one of effective means to protect them against chemical degradation. In this study, the SLNs for all-*trans* retinol (AR) were formulated to improve the stability of AR, whose chemical instability has been a limiting factor in its clinical use. First of all, the physicochemical properties of AR-loaded SLNs, including mean particle diameter and zeta potential, were modulated by changing the total amount of surfactant mixture and the mixing ratio of eggPC and Tween 80 as surfactant mixture. The AR-loaded SLNs formulation was irradiated with a 60-W bulb to investigate the photostability. The extent of photodegradation was measured by high-performance liquid chromatography. The mean particle diameter and zeta potential of the smallest SLNs were 96 nm and −28 mV, respectively. The loading of AR in optimized SLNs formulations rather decelerated the degradation of AR, compared with AR solution dissolved in methanol. Our subsequent study showed that the co-loading of antioxidants greatly enhanced the stability of AR loaded in SLNs, compared with those loaded in SLNs without antioxidant. The photostability at 12 h of AR in SLNs was enhanced folds (43% approximately) higher than that in methanol solution (about 11%). Furthermore, the protecting effect of antioxidants was greatly dependent on the type of antioxidant. Taken together, AR could be effectively stabilized by being loaded in SLNs together with an antioxidant BHT–BHA.

Keywords: All-*trans* retinol; Solid lipid nanoparticles; Stability; Antioxidant

1. Introduction

All-*trans* retinol (AR) is a hydrophobic vitamin A compound that exerts a potent influence on cell differentiation, proliferation, and homeostasis [1,2]. It also helps in maintaining the health of the skin (prevents acne and dermatitis) and surface tissues especially those with mucous linings. Moreover, AR protects skin against skin-aging by neutralizing unstable oxygen molecules (free radical) and participating in the process of collagen propagation. Because of its anti-aging activity, many cosmetic products contain AR as an anti-wrinkle agent. In spite of a wide range of biological and pharmacological effects, the therapeutic and cosmetic uses of AR are still limited due to its poor chemical stability when exposed to air, water or light [3,4]. Therefore, a formulation that can increase the chemical stability of AR needs to be developed.

Recently, increasing attention has been given to solid lipid nanoparticles (SLNs) as a promising carrier system combining several advantages of traditional carriers. Lipid-based carrier systems, such as solid lipid nanoparticle, were developed to overcome some limitations of classical colloidal carriers [5]. Most of all, their components are biodegradable [6] and their toxicity is lower than that of polymeric nanoparticles [7]. They can load sufficient

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amounts of lipophilic drugs [6]. The SLNs can be produced on large industrial scale by high-pressure homogenization and via microemulsions [8]. The solid matrix of SLNs, which can reduce the mobility of loaded drug molecules, improves the stability of drug by protecting the sensitive groups of drug molecules [9]. When optimized, SLNs exhibit high physical stability [10], protection of loaded labile actives against degradation [2], and excellent in vivo tolerability [11]. These several advantages of SLNs have led many research groups to study the applications of SLNs as dosage forms for parenteral [8], oral [12], and topical administrations [13].

We previously reported that SLNs formulation could stabilize all-*trans* retinoic acid, another chemically labile vitamin A analogue [14]. Physicochemical characteristics of SLNs and the chemical stability of loaded drugs are known to be quite dependent on the properties of drugs. With this regard, we aimed to investigate whether stabilization of AR could be achieved by SLNs loading. We report here that co-loading of antioxidant was crucial to stabilize AR by loading in SLNs. Furthermore, we demonstrated that the stability of AR loaded in SLNs was dependent on the type of antioxidants.

2. Materials and methods

2.1. Materials

Solid lipid (DS-CBS) was kindly provided by Doosan Biotech Co. Ltd (Korea). The solid lipid was obtained from crystallization of palm oil by controlling temperature and phase-transfer from solid to liquid at the fixed temperature (37.5 °C). Tween 80 was purchased from Shinyo Pure Chemicals Co. Ltd (Osaka, Japan). Egg phosphatidylcholine (eggPC) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). AR, butylate hydroxyanisol (BHA), butylate hydroxytoluene (BHT), vitamin C, DL-α-tocopherol, and trehalose were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of reagent grade and used without further purification.

2.2. Preparation of AR-loaded SLNs

SLNs were manufactured by hot melt homogenization method [15]. Briefly, 100 mg of solid lipid, 3 mg of AR, and varying amounts of eggPC and Tween 80 were mixed in pear-shaped 25 ml glass tube and then sonicated in bath type sonicator (Branson® ultrasonic cleaner, 3210R-DTH, Branson Ultrasonics Corp., CT, USA) at 60 °C for 2 h. Eight hundred microliters of preheated water (60 °C) was slowly added to the melts (1 g of final total weight) and sonicated in bath type sonicator for 3 h until milky emulsions were obtained. These crude emulsions were homogenized for 4 cycles at 60 °C and 100 mPa using a high-pressure homogenizer (Emulsiflex® EF-B3, Avestin Inc., Canada) wired with heating tape (Thermolyne, Barnstead Int., USA). The homogenized emulsions were cooled in liquid nitrogen and then thawed in water bath at room temperature to produce SLNs.

The antioxidants added AR-loaded SLNs were prepared by using the same method to prepare AR-loaded SLNs. BHT–BHA mixture, vitamin C, and tocopherol were used as antioxidant agents. Each antioxidant (3 mg; in the case of BHT–BHA mixture, each 1.5 mg was mixed) was mixed with 100 mg of solid lipid, 3 mg of AR, 33 mg of Tween 80, and 67 mg of eggPC. Then, following process was identical with the preparation method of AR-loaded SLNs.

2.3. Differential scanning calorimetry

DSC analysis was performed using DSC Q-1000 (TA Instruments, Leatherhead, UK). Two milligrams of sample was weighed in 40 μl aluminum pan. A heating rate of 5 °C/min was employed in the range of 10–100 °C.

2.4. Characterization of AR-loaded SLNs

The mean particle diameter and polydispersity index (P.I.) of AR-loaded SLNs were determined by dynamic light scattering method using electrophoretic light scattering spectrophotometer (ELS-8000, OTSUKA Electronics Co. Ltd., Japan) at a fixed angle of 90° and at 25 °C. The particle diameter analysis data were evaluated using volume distribution to detect even a few large particles. The P.I. is a measure of the distribution of nanoparticle population [16].

The electrophoretic mobility of SLNs was also determined using electrophoretic light scattering spectrophotometer. After dilution of SLNs with distilled water, the electrophoretic mobility was measured. The measured electophoretic mobility data were converted into zeta potential by using Helmholtz–Smoluchowski equation. The processing was done by the software included within the system.

2.5. Loading efficiency of AR loaded in SLNs

After homogenization, the crude AR emulsions were immediately filtered through a 0.45-μm membrane filter to remove precipitated AR [17]. After freezing and thawing, 50 μl of aliquots of the prepared SLNs was dissolved in 950 μl of methanol vortexing for 1 min. Fifty microliters of dissolved solution was directly injected to HPLC and the amount of AR loaded in SLNs was measured by following HPLC analysis method [18]. The HPLC system was consisted of pump (LC-10AS, Shimadzu, Japan), UV detector (SPD-10A, Shimadzu, Japan), and autosampler (717 plus autosampler, Waters, USA). Data were analyzed using dsChrom software (Donam Instruments, Korea). C_{18} reverse-phase column (Capcellpak UG 120, 5 μm, 250 mm × 4.6 mm, Shiseido) was used. The eluent was a mixture of acetonitrile and methanol at the volume ratio of 75:25, and the signal was monitored at 325 nm. The injection volume was 50 μl and the flow rate was 1.0 ml/min. Under this condition, the linear calibration curve of

retinol was obtained in the concentration range of 0.25–100 μg/ml ($r^2 > 0.999$).

2.6. Effect of light on the chemical stability of AR

Samples of AR-loaded SLNs were divided into two groups. One group was taken in the transparent 1.5 ml polypropylene tube and the other group was taken in the lightproof 1.5 ml polypropylene tube (Treff AG, CH-9113 Degersheim, Switzerland). And then, a 60-W bulb (770 Lm) was placed at an 80 cm height from the samples and the samples were illuminated up to 72 h [17]. Fifty microliters of aliquots was taken from each sample at designated time intervals and then dissolved in 950 μl of methanol solution and vortex-mixed for 1 min. Fifty microliters of dissolved solution was injected to HPLC and the amount of intact AR analyzed.

2.7. Statistics

Statistical analysis of data was performed using Student's *t*-test and analysis of variance (ANOVA). A *p*-value of less than 0.05 was considered significant.

3. Results and discussion

For the preparation of AR-loaded SLNs, DS-CBS was selected as a solid lipid component that will constitute the core of SLNs because the lipid has been refined to have a phase transition from solid to liquid at 37.5 °C. EggPC and Tween 80 were chosen as components of surfactant mixture to stabilize SLNs because they are acceptable surfactants even in parenteral administration. With AR-loaded SLNs prepared from these components, their physicochemical characteristics such as the mean particle diameter, polydispersity index (P.I.), and zeta potential were compared as criteria for their physical stability [14,19].

3.1. Optimization of AR-loaded SLNs formulation

Since SLNs prepared with combination of surfactants generally tend to have smaller particle diameter and higher storage stability by preventing particle agglomeration more efficiently [20,21], we prepared AR-loaded SLNs with surfactant mixture composed of varying ratios of Tween 80 and eggPC, and investigated the effect of surfactant composition on the size and zeta potential of resultant SLNs. The mean particle diameter of SLNs prepared with 100% Tween 80 or 100% eggPC was 554 and 364 nm, respectively (Table 1). The combination of eggPC and Tween 80 reduced the particle diameter, resulting in 228 nm in SLNs with 33:67 weight ratio of Tween 80 and eggPC. The P.I. values of AR-loaded SLNs prepared with 100% Tween 80 and 100% eggPC were 0.328 and 0.317. Similar to particle diameter, the P.I. value of SLNs was lowered at the 33:67 weight ratio of Tween 80 and eggPC resulting in 0.198, suggesting narrow size distribution.

Table 1
Effect of content of Tween 80 in the surfactant mixture composed of eggPC and Tween 80 on the mean particle diameter, polydispersity index, and zeta potential of AR-loaded SLNs

Tween 80 in the surfactant mixture (%)	Mean diameter (nm)	Polydispersity index (P.I.)	Zeta potential (mV)
0	364 ± 92	0.317 ± 0.025	−28 ± 3
25	332 ± 7	0.225 ± 0.045	−32 ± 2
33	228 ± 12	0.198 ± 0.024	−22 ± 1
50	359 ± 12	0.278 ± 0.017	−20 ± 1
67	390 ± 34	0.322 ± 0.043	−25 ± 1
75	472 ± 8	0.340 ± 0.025	−28 ± 6
100	554 ± 64	0.328 ± 0.017	−26 ± 4

SLNs were prepared with 3 mg/g of AR and 60 mg of surfactant mixture composed of varying ratios of eggPC and Tween 80. Data represent means ± SD ($n = 3$).

The zeta potential of SLNs was variable in the range of −20 and −32 mV regardless of mixing ratio of eggPC and Tween 80 (Table 1). Previous studies have shown that a minimum zeta potential of greater than −60 mV is required for excellent, of greater than −30 mV for a good physical stability of colloidal carrier systems [22]. In this context, the zeta potential of SLNs prepared with the ratio of 67:33 (w/w) (−22 mV) was only slightly lower than that required to get good electrostatic stabilization.

Taken together, the smallest mean particle diameter and P.I. value of SLNs could be obtained by combination of eggPC and Tween 80 at the ratio of 67:33 (w/w). From these results, the ratio of eggPC–Tween 80 was optimized at 67:33 in the subsequent studies.

3.2. Effect of amount of surfactant mixture

The surfactant amount in SLNs is also an important factor determining the physicochemical characteristics such as mean particle diameter, due to the surface-active properties of surfactants [23]. Therefore, we aimed to investigate whether the increase in the surfactant amount influenced the mean particle diameter of AR-loaded SLNs.

As expected, the mean particle diameter of SLNs tended to decrease with increase in the total amount of surfactant mixture from 60 to 100 mg/g (Fig. 1). The mean particle diameter could be reduced nearby 124 ± 32 nm by preparing SLNs with 100 mg/g surfactant mixture, while that of SLNs with 60 mg/g surfactant mixture was 228 ± 12 nm.

The change of total amount of surfactant mixture did not significantly affect the zeta potential of SLNs (−22 to −28 mV) (Fig. 1). From these results, we fixed the total amount of surfactant mixture as 100 mg/g.

3.3. Thermal analysis of optimized SLNs formulation of AR

With optimized SLNs formulation of AR, we performed thermal analysis to assure the solidification of the lipid core. The melting peak was observed at 34.8 °C (Fig. 2). It was slightly lower than the melting point (37.5 °C) of

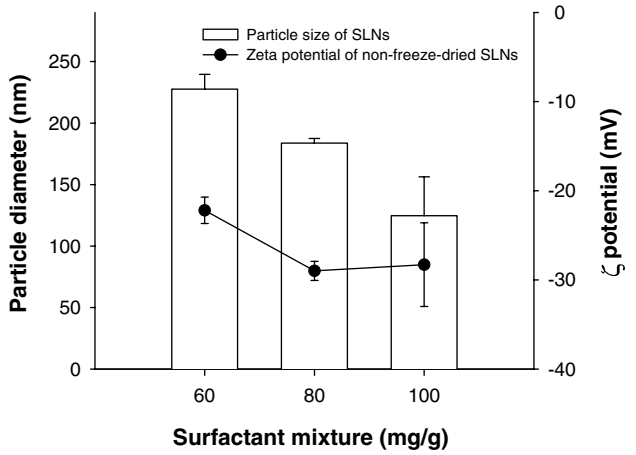

Fig. 1. Effect of total amount of surfactant mixture on the mean particle diameter and zeta potential of AR-loaded SLNs. SLNs were prepared with 60, 80, and 100 mg/g surfactant mixture composed of eggPC–Tween 80 (67:33, w/w). Data represent means ± SD ($n = 3$).

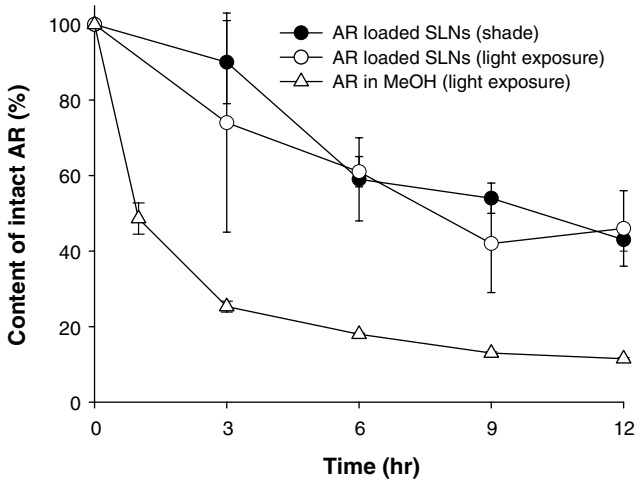

Fig. 3. Effect of light on the chemical stability of AR in SLNs. SLNs were prepared with 3 mg/g of AR, 100 mg of surfactant mixture eggPC–Tween 80 (67:33, w/w). And one group of samples was exposed to light and the other group was shaded from light. Data represent means ± SD ($n = 3$).

bulk DS-CBS, as suggested due to the nanocrystalline size of lipids in SLNs systems [24].

3.4. Chemical stability of AR loaded in SLNs

With optimized SLNs formulation of AR, we investigated the chemical stability of AR by loading in SLNs. The concentration of intact AR in methanol solution rapidly decreased during incubation at room temperature under light exposure (Fig. 3). Within 3 h, less than 30% of AR remained intact in methanol solution. The degradation rate of AR loaded in SLNs was slower than that in methanol solution. However, at every time point, no significant difference in the intact AR content was observed between light-exposed and light-protected AR-loaded SLNs (Fig. 3, $p > 0.05$). For example, after 12 h of incubation, 57% (exposed to light) and 54% (shaded from light) of intact AR in SLNs were degraded. The SLNs formulation was effective in protecting light-induced destabilization of AR, although the decrease in the intact AR content was greatly faster by loading AR in SLNs regardless of light exposure: 46% (exposed to light) and 43% (shaded from light) of AR remained intact after incubation for 12 h of AR-loaded SLNs. These data indicate that SLNs formulation did not significantly protect AR from the degradation by light exposure. Our data are partly inconsistent with the observation of Jenning's [25] studies in which the stabilization of AR was achieved by SLNs loading. With our current data, it remains unclear why our SLNs formulation impaired the stability of AR. It is known that depending on the type of lipids, the lipids constituting SLNs are subjected to auto-oxidation when exposed to air or water [26–28]. It is plausible that lipids used for constituting SLNs in our study may be more prone to auto-oxidation, and thus intact AR loaded in our SLNs formulations may act as an antioxidant scavenging the lipoperoxyl radicals, resulting in the degradation and consumption of AR.

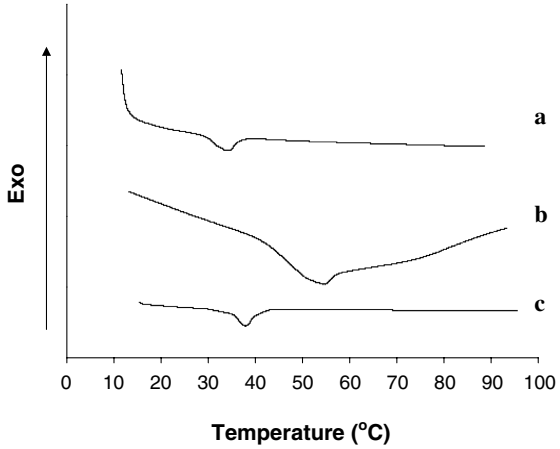

Fig. 2. DSC thermograms of AR-loaded SLNs (a), AR (b), and solid lipid (c).

3.5. Co-loading of AR and antioxidants in SLNs

To check the possibility that the loading of antioxidants may inhibit or retard the degradation of AR loaded in SLNs, we applied three different types of antioxidants for the SLNs preparation by dissolving together in lipid melts (BHT–BHA and tocopherol) or in preheated water (vitamin C).

All three types of antioxidants significantly enhanced the stability of AR. As shown in Fig. 4, after incubation for 48 h under shade, more than 50% of AR remained intact when AR was loaded in SLNs together with antioxidants, while less than 1% was intact in AR loaded in SLNs. These data suggest that the rapid degradation of AR in SLNs was due to the oxidation of AR.

Among three types of antioxidants, BHT–BHA was the most effective in protecting AR from degradation; at 72 h post-incubation, 89%, 48%, and 53% of AR existed as an

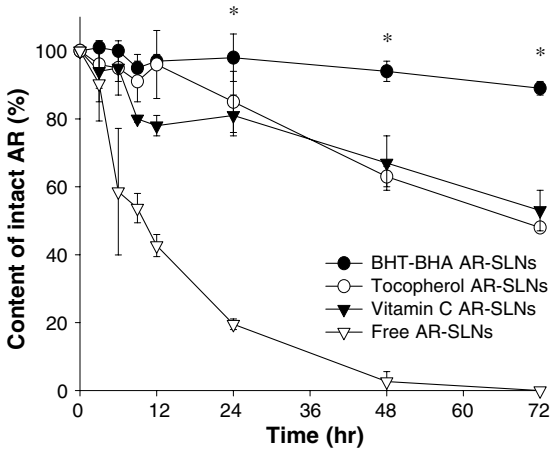

Fig. 4. Effect of antioxidant agents on the chemical stability under shade of AR in SLNs. For the comparison of chemical stability of AR, SLNs were prepared with 3 mg/g of AR, 100 mg of surfactant mixture of eggPC–Tween 80 (67:33, w/w), and 3 mg/g various antioxidants (BHT–BHA mixture, tocopherol, and vitamin C). (*) Significantly different from other groups (ANOVA and Duncan's multiple range test, $P < 0.05$).

intact form when AR was loaded with BHT–BHA, tocopherol, and vitamin C. Under light-exposed conditions, no significant difference was found in the AR stability loaded in SLNs together with BHT–BHA (there were not statistically different, $p > 0.05$), suggesting that the antioxidant

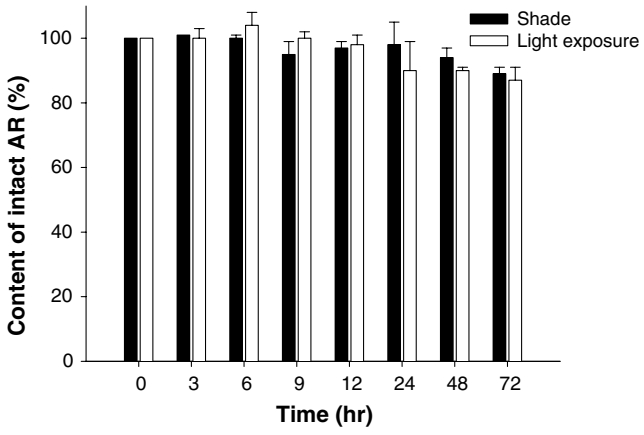

Fig. 5. Effect of light on the chemical stability of AR in BHT–BHA SLNs. Data represent means ± SD ($n = 3$).

did not adversely affect the light-protecting effect of SLNs (Fig. 5).

The inferior potency of vitamin C in protecting AR degradation may be due to the fact that vitamin C is hydrophilic, from which we can expect that it is present in the outer aqueous phase rather than being loaded in the lipid core of SLNs. The superiority of BHT–BHA compared with tocopherol, which are both lipophilic, cannot be explained with our current data. It may be related to the difference in the interaction with AR and antioxidant molecules. In this regard, previous studies have shown that interaction of tocopherol with AR not only prevents the auto-oxidation of AR but also promotes chemical reactivity of AR as a radical scavenger [29].

We showed that loading of antioxidants such as BHT–BHA could enhance the stability of AR, a drug loaded in SLNs. Since loading of antioxidant may also negatively or positively affect the physicochemical characteristics of SLNs, we studied the effect of loading of BHT–BHA on the mean particle diameter, P.I., and zeta potential of SLNs dispersions. As shown in Table 2, the mean particle diameters of the SLNs prepared with BHT–BHA, tocopherol, and vitamin C were not much different from those prepared without antioxidants. The P.I. values of SLNs were also similarly low (0.244–0.283), showing the narrow size distribution. As the zeta potentials of SLNs ranged from −27 to −37 mV, suggesting acceptable electrostatic stability of SLNs. Therefore, the physical properties of SLNs were not affected by adding antioxidants. With regard to the AR loading efficiency, we found that the loading efficiency of SLNs containing BHT–BHA (BHA–BHT SLNs) greatly enhanced the AR loading efficiency, compared with those SLNs prepared without antioxidant co-loading. Alternatively, the agents may be attached to the particle surface and parelectric spectroscopy allows investigating the interaction of substance and the carrier [30]. In future study, the loading and interaction between AR and SLN formulation should be more precisely investigated. These data indicate that the co-loading of BHT–BHA helps the loading of AR in SLNs during preparation of SLNs, as well as playing a role in protecting AR from chemical degradation. Taken together, our results suggest that the stabilization of AR can be achieved by loading in SLNs together with BHT–BHA.

Table 2
Effect of antioxidants on the mean particle diameter, polydispersity index, zeta potential, and loading efficiency of AR-loaded SLNs

Antioxidants	Mean diameter (nm)	Polydispersity index (P.I.)	Zeta potential (mV)	Loading efficiency (%)
None	125 ± 32	0.287 ± 0.035	−28 ± 5	74
BHT–BHA	149 ± 3	0.278 ± 0.024	−31 ± 1	101
Tocopherol	174 ± 6	0.244 ± 0.054	−27 ± 4	90
Vitamin C	172 ± 8	0.283 ± 0.051	−37 ± 2	88

All the SLNs formulations were prepared with 3 mg/g of AR, 100 mg of surfactant mixture composed of eggPC–Tween 80 (67:33, w/w), and 3 mg/g various antioxidants. Data represent means ± SD ($n = 3$).

4. Conclusions

In this study, we showed that AR-loaded SLNs with desirable mean particle diameter, P.I., and zeta potential could be obtained by optimizing the formulation factors such as combination ratio of surfactant mixtures. Although loading of AR in SLNs could not perfectly stabilize AR, the instability of AR could be overcome by co-loading of antioxidants such as BHT–BHA in SLNs. Furthermore, the presence of antioxidants greatly increased the loading efficiency of AR in SLNs. Our study shows that loading of AR in SLNs together with BHT–BHA may provide an effective formulation for AR for the clinical use.

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